Identification and quantitation of all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma

Christian Eckhoff and Heinz Nau

Institut für Toxikologie und Embryonalpharmakologie, Freie Universität Berlin, Garystrasse 5, 1000 Berlin 33, West Germany

Abstract Human plasma was analyzed by high performance liquid chromatography for the presence of retinoic acid and 4-oxoretinoic acid isomers. Peaks that coeluted with the reference compounds all-*trans*-retinoic acid, 13-*cis*-retinoic acid, and 13-*cis*-4-oxoretinoic acid were routinely observed in human plasma. These retinoids were unequivocally identified by the following methods: comigration with reference compounds under several high performance liquid chromatographic conditions; comparison of ultraviolet spectra with those of reference compounds; derivatization with diazomethane and coelution of the methyl esters with reference compounds in a high performance liquid chromatographic system as well as in a gas chromatography system with a mass selective detector. In vitro formation of 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid as artifacts during the analytical procedure was excluded by control experiments. The mean plasma concentrations of the vitamin A metabolites in ten male volunteers were: all-*trans*-retinoic acid: 1.32 ± 0.46 ng/ml; 13-*cis*-retinoic acid: 1.63 ± 0.85 ng/ml; and 13-*cis*-4-oxoretinoic acid: 3.68 ± 0.99 ng/ml. After oral dosing with vitamin A (833 IU/kg body weight) in five male volunteers, mean plasma all-*trans*-retinoic acid increased to 3.92 ± 1.40 ng/ml and 13-*cis*-retinoic acid increased to 9.75 ± 2.18 ng/ml. Maximal plasma 13-*cis*-4-oxoretinoic acid concentrations (average 7.60 ± 1.45 ng/ml) were observed 6 h after dosing which was the last time point in this study. Concentrations of all-*trans*-4-oxoretinoic acid were low or not detectable. Our findings suggest that, in addition to all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid are present in normal human plasma as metabolites of vitamin A. —**Eckhoff, C., and H. Nau.** Identification and quantitation of all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma. *J. Lipid Res.* 1990. **31:** 1445–1454.

Supplementary key words vitamin A metabolism • all-*trans*-retinoic acid • HPLC

Retinol can be metabolized to more polar retinoids such as all-*trans*-RA which has been detected in blood and several tissues of laboratory animals (1–5). All-*trans*-RA cannot be converted back to retinol (6, 7) but can fulfill several important biological functions of vitamin A. These include growth promotion and differentiation of epithelial cells (6–10). A specific cellular binding protein for all-*trans*-RA, CRABP, has been identified (11, 12) and the biological effects of all-*trans*-RA are thought to be mediated by gene-regulating nuclear receptors (13, 14). Therefore, all-*trans*-RA represents a biologically active form of vitamin A. All-*trans*-RA cannot, however, replace retinol in the visual cycle (6) and in reproductive functions (15).

All-*trans*-RA has been found to be present in very small quantities (1–4 ng/ml) in normal human serum or plasma (16–19). 13-*Cis*-RA and the 4-oxo-metabolites of retinoic acid, all-*trans*-4-oxoRA and 13-*cis*-4-oxoRA, have not been unambiguously identified in human blood. In the rat, however, it has been proposed that 13-*cis*-RA is present in the blood and the small intestine under vitamin A steady-state conditions (5). The physiological significance of the presence of two isomers of retinoic acid, all-*trans* and 13-*cis*, is not yet clear.

We investigated human plasma for the presence of endogenous all-*trans*-RA, 13-*cis*-RA, all-*trans*-4-oxoRA, and 13-*cis*-4-oxoRA. Several analytical techniques including HPLC, derivatization, UV spectroscopy, and gas chromatography/selected ion monitoring were used to identify endogenous retinoic acid compounds. Furthermore, we describe a routine HPLC method that was successfully used to investigate the influence of a single dose of vitamin A (833 IU/kg body weight) on the retinoic acid levels in plasma.

Abbreviations: CRABP, cellular retinoic acid binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC, high performance liquid chromatography; RA, retinoic acid; TBAHS, tetrabutylammoniumhydrogensulfate; UV, ultraviolet.

MATERIALS AND METHODS

Chemicals

All-*trans*-RA and 13-*cis*-RA were purchased from Sigma (Munich, F.R.G.). All-*trans*-4-oxoRA, 13-*cis*-4-oxoRA, and RO 13-7652 (13-*cis*-acitretin; (2Z, 4E, 6E, 8E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid) were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Lyophilized analytical grade bovine serum albumin and HEPES buffer were from Serva (Heidelberg, F.R.G.) TBAHS was from Fluka (Buchs, Switzerland). Methanol was HPLC grade and was supplied, together with all other analytical grade chemicals, from Merck (Darmstadt, F.R.G.). Water for HPLC was prepared by a Milli-Q-water purification system. Diazomethane was produced from 1-methyl-3-nitro-1-nitrosoguanidine (MNNG, Aldrich) with a MNNG-diazomethane kit.

Laboratory precaution

All handling of retinoids and samples was performed in a room with dim yellow light.

Solutions

Stock solutions of 13-*cis*-4-oxoRA, all-*trans*-4-oxoRA, 13-*cis*-RA, all-*trans*-RA, and the internal standard RO 13-7652 were prepared by dissolving 10 mg of each compound in 100 ml ethanol, respectively. All stock solutions were kept at − 25°C. The internal standard working solution was prepared by adding 15 μl of RO 13-7652 solution to 35 ml acetonitrile and adding 65 ml of an aqueous 2% ammonium acetate solution.

Plasma samples

Blood was drawn from healthy male volunteers and plasma was prepared by centrifugating the blood in heparinized tubes for 10 min at 1500 *g* and 4°C. The samples were stored in polypropylene tubes at − 80°C until analysis.

Dosing of vitamin A

Volunteers received retinyl palmitate (833 IU/kg body weight) as oily drops (A-Vicotrat oleosum forte, Heyl, Berlin, F.R.G.).

Sample preparation

Samples were prepared for injection into the HPLC system by a solid phase extraction technique using the Varian AASP module. This is an automated device for solid phase extraction followed by HPLC analysis (20). An AASP C2 cartridge (silica modified with ethyl groups) was pre-conditioned with 1.5 ml methanol followed by 0.6 ml 2% ammonium acetate solution. Plasma (0.35 ml, centrifuged at 2000 *g* and 4°C for 10 min) was diluted with 2% ammonium acetate solution (0.6 ml) and the internal standard working solution (0.6 ml) was added. This mixture was pressed through the cartridge where lipophilic plasma components were transferred to the modified silica. The cartridge was washed twice with 1.5 ml 0.5% aqueous ammonium acetate solution–acetonitrile 8.5:1.5 to remove proteins and other interfering polar substances. The cassette was then loaded onto the AASP. Each cartridge was purged with 0.5 ml 0.5% aqueous ammonium acetate solution–methanol 8.5:1.5 prior to on-line elution onto the analytical column in order to remove air from the cartridge. AASP conditions were: run time 12 min; cycle time 15 min; valve reset 3.7 min. At the end of the run time, the cartridge was again purged with 0.5 ml 0.5% aqueous ammonium acetate solution–methanol 8.5:1.5 to remove all residual analytical eluent from the AASP capillaries.

Chromatography

HPLC system I was designed for the analysis of plasma samples. It consisted of two pumps (type 64), a gradient programmer 50B, a dynamic mixing chamber (all from Knauer, Berlin, F.R.G.), an AASP sample processor (Varian, Darmstadt, F.R.G.), and a SPD-6A variable wavelength UV-detector (flow cell: 10 × 1 mm) and a C-R4A two-channel integrator (Shimadzu). The column (120 × 4 mm) was packed with Spherisorb ODS II 3 μm (Phase Separations) in our laboratory and placed in a TC 931 column block heater (Applied Chromatography Systems Ltd., Macclesfield, U.K.). A binary gradient was formed from solvent A: 60 mM ammonium acetate, adjusted to pH 5.75 with acetic acid–methanol 1:1 and solvent B:pure methanol. The solvents were degassed by ultrasonic treatment prior to their use. The gradient conditions were (only the percent solvent B are mentioned): I) 15% solvent B at the time of injection (i.e., switching of the AASP 10 way valve); II) linear increase to 90% solvent B at 11 min; III) linear increase to 99% solvent B at 11.2 min; IV) maintenance of 99% solvent B until 12 min. The starting conditions of the gradient (15% solvent B). were reached again at 12.5 min. The flow rate was 0.7 ml/min and the column was heated to 60°C. UV detection was carried out at 340 nm. **Fig. 1A** shows the chromatogram of a standard sample.

HPLC system II was used for rechromatography of peaks collected from system I. The apparatus consisted of one type 64 Knauer pump, a Rheodyne 7125 injector equipped with a 200 μl sample loop, a TC 950 column block heater, and a SPD-6A UV-detector. Chromatograms were recorded on a C-R4A integrator. A peak eluate (ca. 150 μl) was directly injected on a 120 × 4 mm Nucleosil 5 C18 column (Macherey und Nagel, Düren, F.R.G.) and eluted with 1.2 ml/min 10 mM TBAHS, 20 mM

HEPES (pH 7.0)–methanol 3.5:6.5 (system IIa) or 1:4 (system IIb) at 35°C. The retention time was compared to that of the appropriate standard.

HPLC system III was used to chromatograph the methylated retinoic acid derivatives and was identical with system II except for a 300 × 4 mm Hypersil ODS 5 μm column (Shandon) which was eluted with 1.2 ml/min 60 mM ammonium acetate (pH 5.75)–methanol 1:4 (system IIIa) or 1:9 (system IIIb) at 30°C. For derivatization, a peak eluate was diluted with water and extracted on an AASP C8 cartridge. After washing with 1 ml 20% methanol in water, the compound was eluted with 0.5 ml methanol and treated with ca. 40 μmol diazomethane dissolved in 0.25 ml diethylether. After evaporation of the solvents under nitrogen, the methylated product was injected in 100 μl of mobile phase into HPLC system IIIa or IIIb. The retention time was compared to that of a methylated retinoid standard.

Gas chromatography/selected ion monitoring

The system consisted of a 5890 gas chromatograph combined with a 5970 mass selective detector (Hewlett Packard). One μl samples were injected (injector temperature: 250°C for 13-*cis*- and all-*trans*-methylretinoate and 300°C for 13-*cis*-4-oxomethylretinoate) on a 0.2 mm × 20 m DB-5-30W column. Helium was the carrier gas (5 psi at column head). The oven program was: initial temperature 180°C, after 3 min increasing with 15°C/min to 240°C. Chromatograms were recorded in the single ion mode with the detector focused on *m/z* 328 or *m/z* 314.

Control experiments

To exclude the possibility of artifact formation, a small amount of either 13-*cis*-RA or all-*trans*-RA reference compound, dissolved in 50% methanol, was added to freshly drawn whole blood (5 ng/ml of blood). Plasma was prepared by centrifugation and submitted to HPLC analysis. Aliquots of the prepared plasma samples were frozen at −25°C and analyzed after 14 weeks of storage.

Calibration, quantitation

Calibration was done by analyzing a known amount of the reference retinoids (usually 25 ng/ml, respectively) dissolved in a retinoid-free 5% solution of pure bovine serum albumin in phosphate-buffered saline. RO 13-7652 was used as an internal standard. The calibration factors were calculated from the peak area ratios. The limit of quantitation was 0.5 ng/ml at a signal-to-noise ratio of 5:1.

RESULTS

HPLC analysis of normal human plasma

Fig. 1B shows the chromatogram of a plasma sample from a healthy male volunteer who had fasted overnight. IS marks the internal standard RO 13-7652. Letters A, B, and C indicate peaks that coeluted with the reference compounds 13-*cis*-4-oxoRA (2), 13-*cis*-RA (3), and all-*trans*-RA (4) (Fig. 1A). Changes of the concentration of ammonium acetate or the pH in the eluate selectively influenced the retention times of peaks A, B, and C, whereas other sample components were virtually not affected. Decreasing the concentration of ammonium acetate and/or increasing the pH by the addition of ammonia resulted in shorter retention times. Conversely, the increase of the concentration of ammonium acetate and/or the decrease of the pH by adding acetic acid led to prolonged retention times. Under all conditions, the retention times of the reference compounds 13-*cis*-4-oxoRA, 13-*cis*-RA, and all-*trans*-RA were changed in the same manner and extent as was the case for peaks A, B, and C (data not shown).

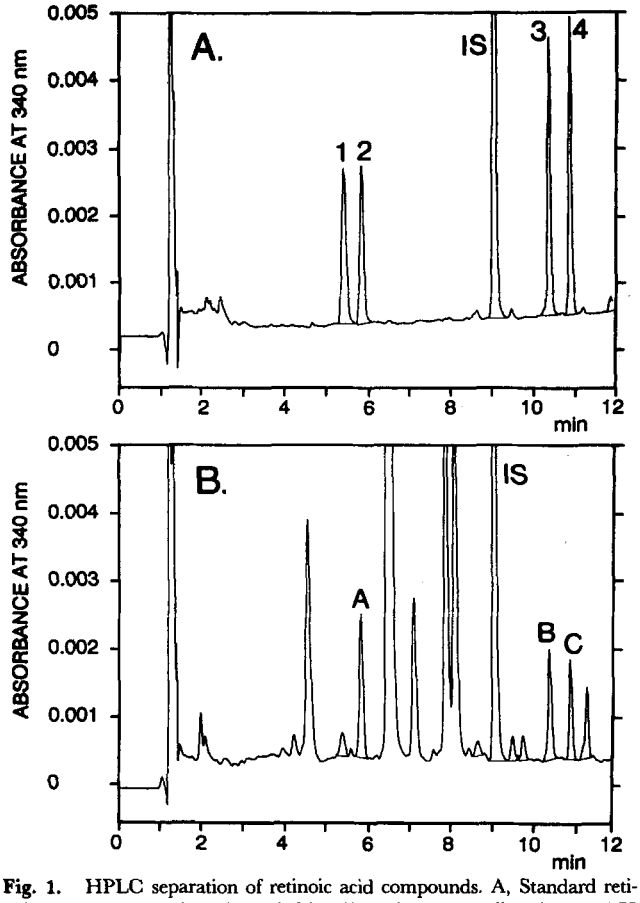

Fig. 1. HPLC separation of retinoic acid compounds. A, Standard retinoids at a concentration of 5 ng/ml in 1% bovine serum albumin (= 1.75 ng of each compound injected). 1, All-*trans*-4-oxoRA (5.43 min); 2, 13-*cis*-4-oxoRA (5.85 min); 3, 13-*cis*-RA (10.41 min); 4, all-*trans*-RA (10.91 min); IS, internal standard RO 13-7652 (9.08 min). B, Plasma sample taken from a male volunteer. Retention times of the indicated peaks were: A, 5.84 min; B, 10.41 min; C, 10.91 min; IS, 9.09 min. The small peak eluting before peak A at 5.40 min could not be identified as all-*trans*-4-oxoRA by the described methods. Furthermore, it was not enhanced by a single oral dose of 833 IU of vitamin A/kg body weight.

Rechromatography

The addition of quaternary ammonium salts such as tetrabutylammonium to the mobile phase results in an in-

crease in retention times of organic acids in reversed phase chromatography (21). The chromatographic behavior of the reference retinoids and the collected eluates from peaks A, B, and C were investigated under ion-pair conditions. The HPLC systems used (systems IIa and IIb) were based on a HPLC separation that was successfully used for the analysis of retinoic acid in animal blood and tissues (22). Coelution of the reference compounds 13-*cis*-4-oxoRA with peak A eluate, 13-*cis*-RA with peak B eluate, and all-*trans*-RA with peak C eluate was observed (**Fig. 2**).

UV-spectra

The UV spectra of the very low concentrations of the putative retinoic acid compounds in plasma were attained by repeated analysis of a pooled plasma sample at various wavelength settings. A standard made with 15 ng of each retinoid/ml of a 5% bovine serum albumin solution was analyzed to obtain the authentic retinoid spectra. Very similar absorption maxima were found for peak A and authentic 13-*cis*-4-oxoRA (**Fig. 3A**, λ_{max} ca. 360 nm), peak B and 13-*cis*-RA (Fig. 3B, λ_{max} = 340 nm), and peak C and all-*trans*-RA (Fig. 3C, λ_{max} = 340 nm).

Derivatization

Derivatization with diazomethane has previously been used in identification of retinoic acid derivatives (16, 23). Furthermore, methylation of retinoic acid is a necessary procedure to obtain a derivative that can be analyzed by gas chromatography. Methylated peak A comigrated in HPLC system IIIa with 13-*cis*-4-oxomethylretinoate; in HPLC system IIIb, peak B methylester and peak C methylester comigrated with 13-*cis*-methylretinoate and all-*trans*-methylretinoate, respectively (**Fig. 4**).

Gas chromatography/selected ion monitoring

Gas chromatography is an additional and useful approach to compare the identity of a sample component with a reference compound. This is particularly true when a mass selective detector is used. Focused on the molecular weight of 13-*cis*-4-oxomethylretinoate (*m/z* 328), peak A methylester exhibited an intense signal with the retention time of the standard 13-*cis*-4-oxomethylretinoate (**Fig. 5**). The analysis of peak B methylester and peak C methylester with detection at *m/z* 314 (molecular weight of 13-*cis*-methylretinoate and all-*trans*-methylretinoate) demonstrated two peaks that coeluted with the standard compounds 13-*cis*-methylretinoate and all-*trans*-methylretinoate, respectively (**Fig. 6**).

Control experiments

The addition of either 13-*cis*-RA or all-*trans*-RA reference compound to freshly drawn whole blood resulted in a selective increase of the concentration of the added com-

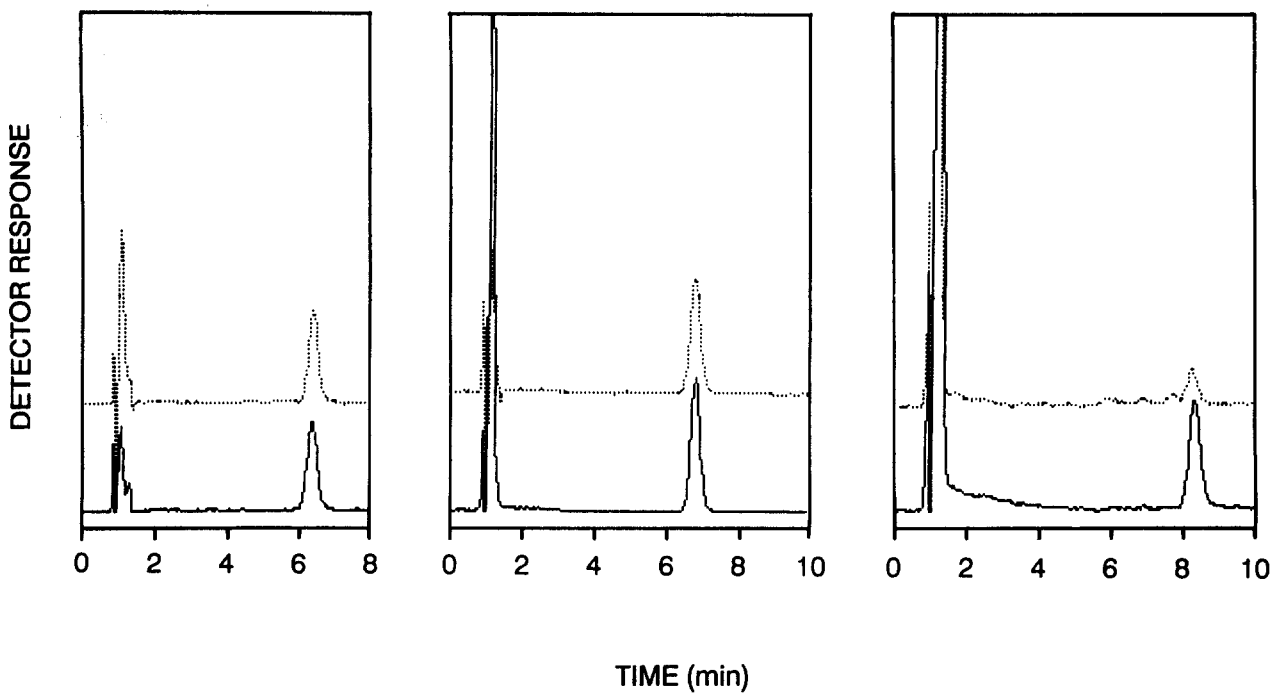

Fig. 2. Rechromatography of peak eluates A, B, and C in an ion pair HPLC system and comparison of the chromatographic behavior with that of reference compounds. The reference chromatograms are drawn with solid lines (from left to right, 13-*cis*-4-oxoRA, 13-*cis*-RA, all-*trans*-RA). The overlay chromatograms drawn with dotted lines show from left to right; peak A eluate, peak B eluate, and peak C eluate. UV detection was carried out at 356 nm (system IIa, left) or 340 nm (system IIb, middle and right). The peak retention times were: 13-*cis*-4-oxoRA 6.46 min, peak A eluate 6.47 min; 13-*cis*-RA 6.82 min, peak B eluate 6.77 min; all-*trans*-RA 8.33 min, peak C eluate 8.27 min.

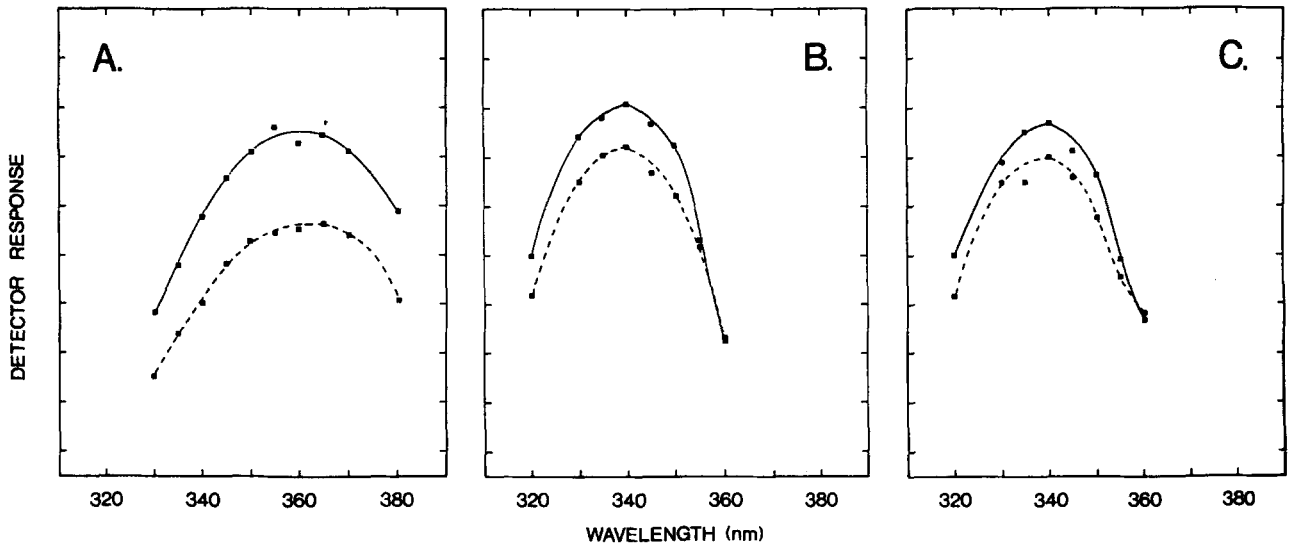

Fig. 3. UV spectra of retinoids. The detector response (integrated peak area, arbitrary units) was plotted against wavelength settings at which an albumin standard containing the authentic retinoids (solid lines) or a pooled plasma sample (broken lines) were repeatedly analyzed. A, Reference compound 13-*cis*-4-oxoRA and peak A from plasma. B, Reference compound 13-*cis*-RA and peak B from plasma. C, Reference compound all-*trans*-RA and peak C from plasma.

pound in the resulting plasma sample (**Table 1**). This was also true when the prepared plasma was analyzed after 14 weeks of storage at −25°C (Table 1). All individual retinoic acid compounds including the internal standard were completely stable on the AASP C2 cartridge for at least 6 h after plasma extraction.

Recovery, reproducibility

Recovery of the retinoids from a spiked human plasma was determined by comparison of the peak areas in the spiked plasma minus the endogenous peak areas of the plasma used for spiking with the peak areas obtained after direct injection of respective amounts of retinoids in 50% methanol into the sample loop of the AASP 10-way valve. For all analytes, recovery was quantitative (>98%) at concentrations of 10 and 100 ng/ml. The excellent recovery shows the efficiency of the solid phase extraction even in the case of 13-*cis*-RA, which is known to be 99.9% bound to albumin in human plasma (24). It was essential, however, to sufficiently dilute the plasma sample with buffer and to add some organic water-miscible solvent such as alcohol or acetonitrile prior to the extraction (25). Be-

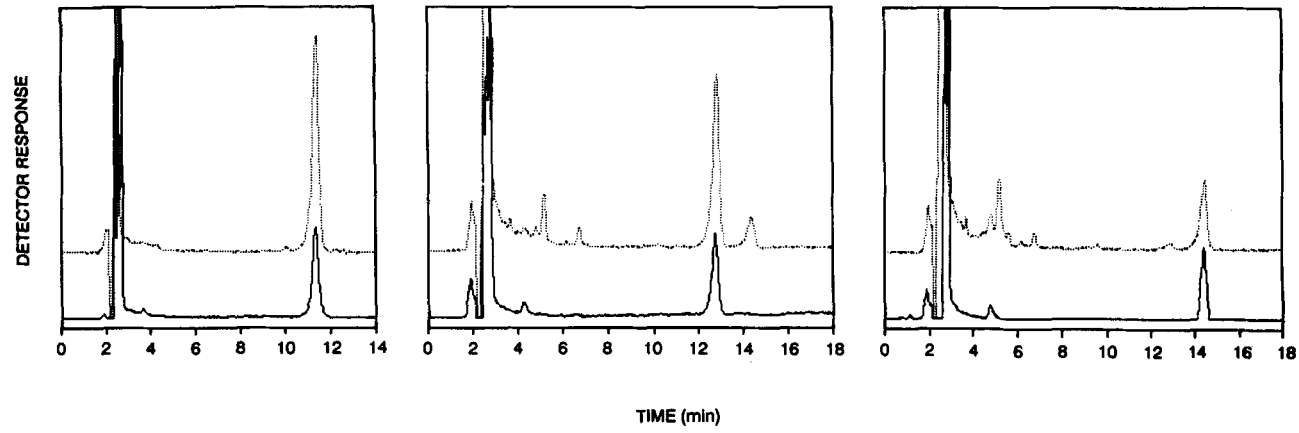

Fig. 4. Chromatography of the methylated peak eluates A, B, and C in a reversed phase HPLC system and comparison of the chromatographic behavior with that of reference compounds. The reference chromatograms are drawn with solid lines (from left to right, 13-*cis*-4-oxomethylretinoate, 13-*cis*-methylretinoate, all-*trans*-methylretinoate). The overlay chromatograms drawn with dotted lines show from left to right: methylated peak A eluate, methylated peak B eluate, and methylated peak C eluate. UV detection was carried out at 356 nm (system IIIa, left) or 340 nm (system IIIb, middle and right). The peak retention times were: 13-*cis*-4-oxomethylretinoate 11.31 min, peak A methylester 11.33 min; 13-*cis*-methylretinoate 12.79 min, peak B methylester 12.82 min; all-*trans*-methylretinoate 14.37 min, peak C methylester 14.37 min. During the methylation of peak B eluate, some all-*trans*-methylretinoate was formed in addition to 13-*cis*-methylretinoate. The same phenomenon had been observed when analyzing methylated peak B eluate with gas chromatography/selected ion monitoring (Fig. 6, middle).

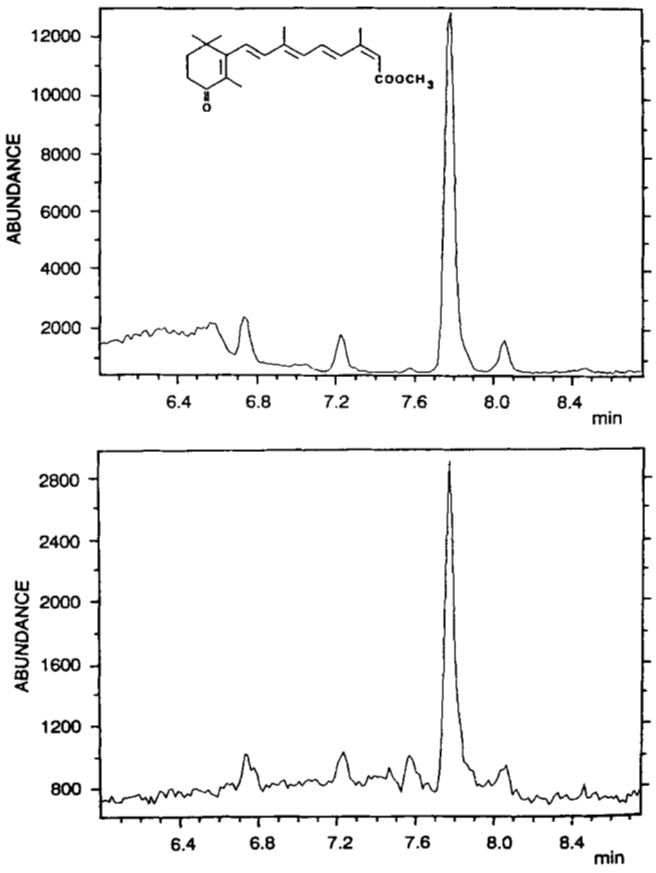

Fig. 5. Selected ion monitoring (m/z = 328) of 13-*cis*-4-oxomethylretinoate. Top, 10 ng of reference compound. Bottom, methylated peak A eluate. No peaks eluted prior to 6 min.

cause the sample reservior of the AASP prep station has a volume of 1.6 ml, 0.35 ml of plasma was the maximum volume that could be quantitatively extracted. All recovery data are presented in **Table 2**. Recovery was also determined from a spiked solution of pure 5% bovine serum albumin in phosphate-buffered saline. There was no significant difference in the recovery of the retinoids from human plasma (data not shown). This allowed the use of the bovine serum albumin solution as a retinoid-free substitute for human plasma during calibration.

Reproducibility was determined by repetitive measurements of a plasma to which 5 ng/ml 13-*cis*-4-oxoRA, all-*trans*-4-oxoRA, 13-*cis*-RA, and all-*trans*-RA, respectively, had been added. From the results presented in **Table 3** it seems also possible to run the analysis without internal standardization since the reproducibility was not diminished under external standard conditions.

Retinoic acid concentrations in human plasma

Retinoic acid concentrations were determined in plasma from untreated humans (normal plasma) and in plasma from volunteers dosed with 833 IU of vitamin A/kg.

Table 4 shows the mean concentrations of the identified retinoic acid compounds in human plasma. The plasma concentration of 13-*cis*-4-oxoRA in untreated subjects was

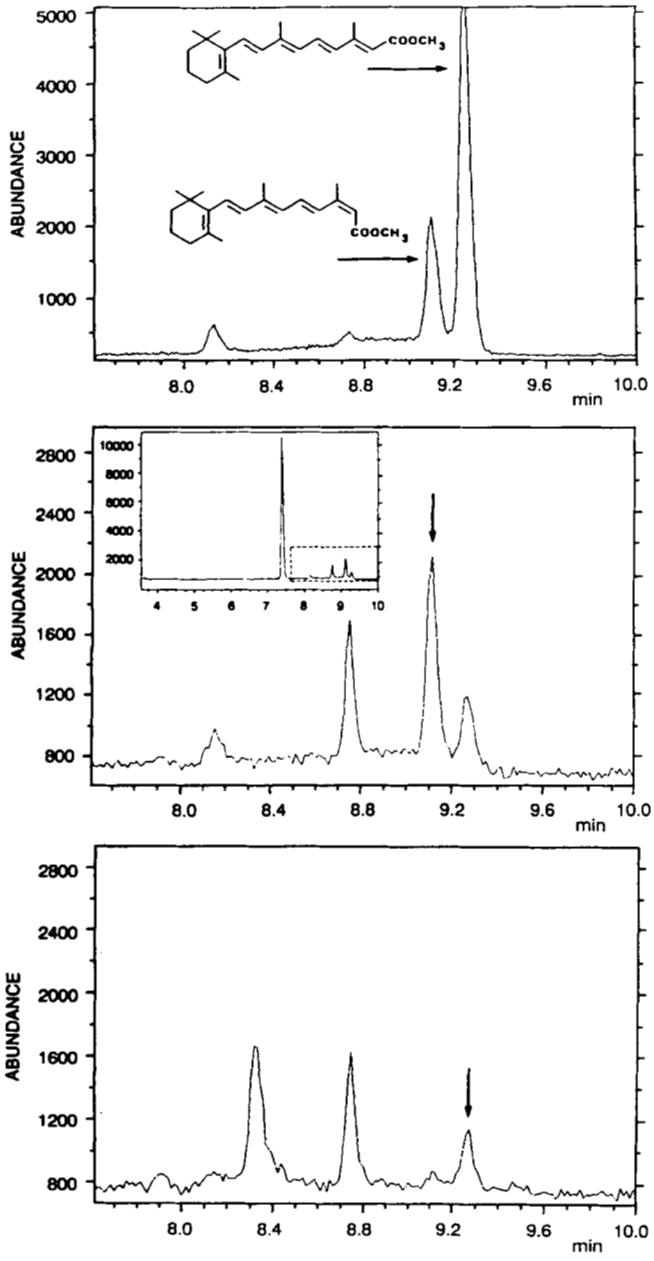

Fig. 6. Selected ion monitoring (m/z = 314) of 13-*cis*-methylretinoate and all-*trans*-methylretinoate. Top, 5 ng of 13-*cis*-methylretinoate and 10 ng of all-*trans*-methylretinoate reference compounds. No peaks eluted prior to 7.6 min. Middle, methylated peak B eluate. The broken line in the inset shows the part of the chromatogram that is scaled up. Some isomerization from 13-*cis*-methylretinoate to all-*trans*-methylretinoate occurred during the derivatization. The same phenomenon had been observed in the HPLC analysis of the methylated peak B eluate (Fig. 4, middle). The large peak eluting at 7.37 min was identified, by recording a full spectrum, as methyl dehydroabietate (mol wt = 314), which is commonly used as a plastic additive (The Merck Index, Ninth Edition). Bottom, methylated peak C eluate. The impurity at 7.37 min was also present in this sample (not shown).

TABLE 1. Control equipment

Sample	13-*Cis*-4-oxoRA	All-*trans*-4-oxoRA	13-*Cis*-RA	All-*trans*-RA
		ng/ml plasma		
Original blood	4.17	-	2.27	1.71
+ 13-*cis*-RA	4.10	-	9.32	1.70
	(4.35)[a]	(-)	(9.68)	(1.82)
+ All-*trans*-RA	4.03	-	2.27	7.94
	(4.36)	(-)	(2.46)	(8.59)

The values shown are ng/ml of plasma prepared from a blood sample that was supplemented with approximately 5 ng/ml of one reference retinoid where indicated; -, not found.
[a]Values in parentheses indicate the concentrations measured after 14 weeks of storage of the plasma at −25°C.

higher than 13-*cis*-RA and all-*trans*-RA concentrations, and 13-*cis*-RA tended to be higher than all-*trans*-RA. Vitamin A intake significantly increased the concentrations of 13-*cis*-4-oxoRA, 13-*cis*-RA, and all-*trans*-RA. 13-*Cis*-RA increased sixfold over the basal concentration, whereas all-*trans*-RA increased only threefold. Individual maxima occurred between 1.5 and 3.75 h after dosing for 13-*cis*-RA and between 2 and 4.5 h for all-*trans*-RA. 13-*Cis*-4-oxo-RA did not reach a maximum until 6 h after dosing.

DISCUSSION

The biologically active vitamin A metabolite all-*trans*-RA has previously been shown to be an endogenous compound of human blood (16–19). The basic new finding of the present study is that 13-*cis*-RA and 13-*cis*-4-oxoRA were also unambiguously identified in blood from normally nourished men who had not been exposed to isotretinoin. Several analytical procedures have been used to clearly demonstrate these findings. The procedures included comigration with reference retinoids in various HPLC systems both before and after derivatization, UV spectroscopy, and gas chromatography with a mass selective detector. Mass spectrometry was achieved in the single ion monitoring mode since the sensitivity of this method was much higher than a scanning method. Considering the consistent results of five different analytical procedures, we are confident that peaks A, B, and C derived from human plasma samples are identical to the reference compounds 13-*cis*-4-oxoRA, 13-*cis*-RA, and all-*trans*-RA, respectively.

We used a solid-phase extraction technique that offered the advantage that the retinoids were never exposed to high concentrations of organic solvents. Mass-transfer of the retinoids from the sample to the stationary phase of the AASP C2 cartridge took place in a diluted plasma milieu. In this environment, the stereochemical stability of 13-*cis* or all-*trans* configured retinoids is very high (26). The AASP solid-phase extraction also avoids evaporation steps that can lead to retinoid instability. Control experiments confirmed these considerations. We found a high stereochemical stability of 13-*cis*-RA and all-*trans*-RA during plasma preparation, storage of frozen plasma, and the entire analytical procedure. Thus, in vitro formation of 13-*cis*-RA and 13-*cis*-4-oxoRA from all-*trans*-RA during the analytical procedure can be excluded.

TABLE 2. Recovery of 13-*cis*-4-oxoRA, all-*trans*-4-oxoRA, 13-*cis*-RA and all-*trans*-RA from spiked human plasma

Compound[a]	Concentration	% Recovery (n = 6)
	ng/ml	*mean ± SD*
All-*trans*-4-oxoRA	10	99.8 ± 1.8
	100	98.0 ± 1.1
13-*Cis*-4-oxoRA	10	102.4 ± 1.4
	100	100.5 ± 1.0
13-*Cis*-RA	10	101.5 ± 0.9
	100	99.2 ± 1.3
All-*trans*-RA	10	101.5 ± 0.9
	100	98.3 ± 1.6

[a]The compounds are listed in the order of their retention times.

TABLE 3. Intra- and inter-assay reproducibility expressed as coefficients of variation (%) of a human plasma sample spiked with 5 ng/ml of each compound

	Internal Standardization		External Standardization	
Compound[a]	Intra-assay (n = 9)	Inter-assay (n = 9)	Intra-assay (n = 9)	Inter-assay (n = 9)
		%		
All-*trans*-4-oxoRA	5.0	5.4	4.0	5.2
13-*Cis*-4-oxoRA	2.6	3.6	2.2	3.4
13-*Cis*-RA	1.8	4.7	2.3	5.3
All-*trans*-RA	5.7	7.3	5.5	8.5

[a]The compounds are listed in order of their retention times.

TABLE 4. Retinoic acid concentrations in human plasma

Sample	n[a]	Concentration		
		13-*Cis*-4-oxo-RA	13-*Cis*-RA	All-*trans*-RA
		ng/ml plasma, mean ± SD		
Normal plasma	10	3.68 ± 0.99	1.63 ± 0.85	1.32 ± 0.46
Max. plasma conc. after 833 IU vitamin A/kg body weight	5	7.60 ± 1.45[b,c]	9.75 ± 2.18[c,d]	3.92 ± 1.40[c]

[a]Number of subjects.
[b]Highest concentration measured until 6 h after dosing.
[c]Different versus normal plasma at $P < 0.01$ (Student's *t*-test).
[d]Different versus maximum of all-*trans*-RA at $P < 0.01$ (Student's *t*-test).

It has recently been suggested, that all four retinoids including all-*trans*-4-oxoRA were present at similar concentrations in a human control plasma (25). However, the described method (25) was designed for the determination of the metabolism of therapeutic doses of isotretinoin (13-*cis*-RA) and peak identity was confirmed by coelution with reference compounds in one HPLC system only. The presence of all-*trans*-4-oxoRA in normal human plasma was not confirmed by our results. Only in one volunteer after chronic exposure to 833 IU of vitamin A/kg once daily, did we observe some all-*trans*-4-oxo-RA (Eckhoff, C., and H. Nau, unpublished observation). The lack or the very low concentrations of all-*trans*-4-oxoRA in human control plasma can be explained by the observation of Frolik et al. (23) who found that low, physiological concentrations of 13-*cis*-RA and all-*trans*-RA seem to have only one common 4-oxo-metabolite which is 13-*cis*-oxoRA.

The observed concentrations of all-*trans*-RA in normal subjects (Table 1) were in good agreement with data published by Barua and Olson (19) who found an average all-*trans*-RA concentration of 1.80 ± 0.80 ng/ml of serum. Other groups (16–18), however, found higher all-*trans*-RA concentrations (ca. 4 ng/ml). In contrast to the methods of these other groups, Barua and Olson (19) did not use hydrochloric acid or sodium hydroxide for the sample extraction. Since all-*trans*-retinoyl β-glucuronide was also found to be an endogenous compound of human blood (19), it was suggested that the use of strong acid or alkali could hydrolyze some of the all-*trans*-retinoyl β-glucuronide, thus resulting in higher values of all-*trans*-RA. In most subjects that we studied to date, 13-*cis*-RA had a slightly higher concentration than all-*trans*-RA. Statistical evaluation of the present data, however, showed no significant difference ($P > 0.05$, Student's *t*-test) between the

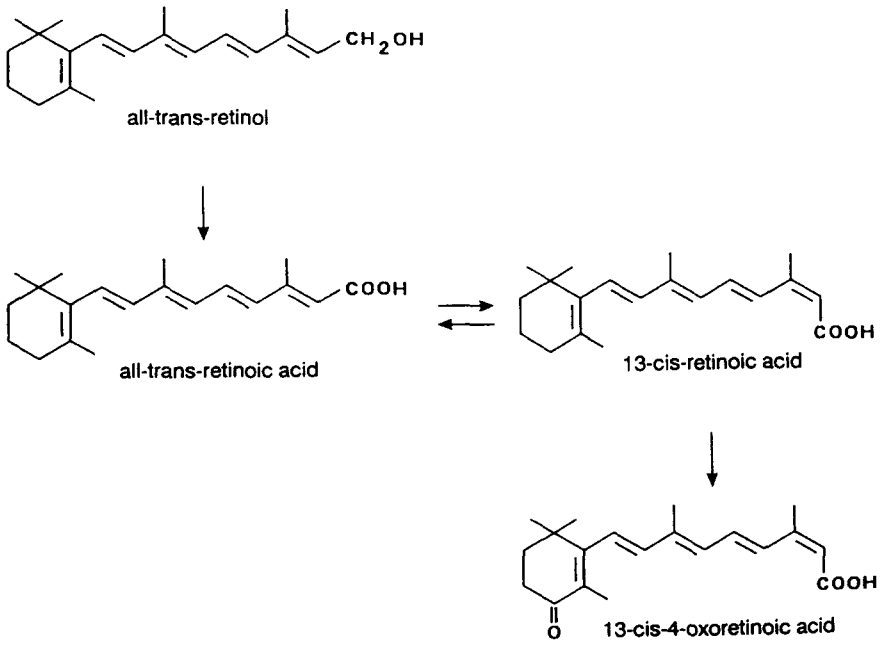

Fig. 7. Proposed metabolic pathways by which physiological doses of vitamin A can result in higher concentrations of 13-*cis*-RA and 13-*cis*-4-oxoRA compared to all-*trans*-RA in human plasma.

plasma concentrations of 13-*cis*-RA and all-*trans*-RA. However, after dosing with vitamin A (833 IU/kg body weight) the mean maximal plasma concentration of 13-*cis*-RA (9.75 ± 2.18 ng/ml) was significantly higher than the mean maximal plasma concentration of all-*trans*-RA (3.92 ± 1.40 ng/ml). Furthermore, a significant increase in the mean maximal plasma concentration of 13-*cis*-4-oxo RA (7.60 ± 1.45 ng/ml) was observed while all-*trans*-4-oxoRA remained undetectable (<0.5 ng/ml). The generation of such quantities of 13-*cis*-configured retinoids from all-*trans* retinol was unexpected and brings up the question of whether 13-*cis*-RA is only a detoxification product of all-*trans*-RA or whether there is an independent role for 13-*cis*-RA as a biologically active metabolite of vitamin A. **Fig. 7** is a proposal for the metabolic pathways likely to be involved in the generation of 13-*cis* configured retinoids from all-*trans*-retinol.

The presented HPLC method appears to be a powerful tool for investigations concerning vitamin A metabolism. With its low limit of quantitation (0.5 ng/ml) it allows the determination of physiological concentrations of 13-*cis*-4-oxoRA, 13-*cis*-RA, and all-*trans*-RA in human plasma. Sample preparation with the Varian AASP is extremely easy and, together with a quick HPLC elution, allows the analysis of a large series of samples in a short time. Separation of the retinoids was achieved using a reversed phase system with an ammonium acetate buffer. Mixtures of aqueous ammonium acetate and acetonitrile or methanol are commonly used by many investigators for the analysis of retinoic acid compounds (27). We used a 3 μm octadecyl silica column with column heating and a steep gradient which resulted in narrow, well-resolved peaks. For routine analysis, we use a second UV-detector (Shimadzu SPD 6-AV; flow cell: 10 × 1 mm) operating at 356 nm which is connected in series to the first detector which operates at 340 nm. Outputs of both detectors are independently processed by the C-R4A two-channel integrator. This adds the characteristics UV-absorbance ratio of the retinoid peaks as a second argument for a correct peak identification to the peak retention time argument. The assay method is successfully used for studies on the vitamin A metabolism in human as well as in monkey plasma.

We thank Uwe Noetzel, Hewlett-Packard GmbH, Hamburg, for an invitation to use his GC-MS device and for his help with the system. We are grateful to Werner Wittfoht for his technical assistance, Ralf-Siegbert Hauck for drawing the structural formulas, and William Slikker, Jr., for valuable suggestions on the manuscript.

Manuscript received 27 November 1989 and in revised form 23 April 1990.

REFERENCES

1. Emmerick, R. J., M. H. Zile, and H. F. De Luca. 1967. Formation of retinoic acid from retinol in the rat. *Biochem. J.* **102:** 606–611.
2. Kleiner-Bössaler, A., and H. F. De Luca. 1971. Formation of retinoic acid from retinol in the kidney. *Arch. Biochem. Biophys.* **142:** 371–377.
3. Ito, Y. L., M. H. Zile, H. Ahrens, and H. F. De Luca. 1974. Liquid-gel partition chromatography of vitamin A compounds: formation of retinoic acid from retinyl acetate in vivo. *J. Lipid Res.* **15:** 517–524.
4. McCormick, A. M., and J. L. Napoli. 1982. Identification of 5,6-epoxyretinoic acid as an endogenous retinol metabolite. *J. Biol. Chem.* **257:** 1730–1735.
5. Cullum, M. E., and M. H. Zile. 1985. Metabolism of all-*trans*-retinoic acid and all-*trans*-retinyl acetate. *J. Biol. Chem.* **260:** 10590–10596.
6. Dowling, J. E., and G. Wald. 1960. The biological function of vitamin A acid. *Proc. Natl. Acad. Sci. USA.* **46:** 587–608.
7. Krishnamurthy, S., J. G. Bieri, and E. L. Andrews. 1963. Metabolism and biological activity of vitamin A acid in the chick. *J. Nutr.* **79:** 503–510.
8. Arens, J. F., and D. A. van Dorp. 1946. Activity of "vitamin A-acid" in the rat. *Nature.* **158:** 622–623.
9. Zile, M. H., and H. F. De Luca. 1968. Retinoic acid: some aspects of growth-promoting activity in the albino rat. *J. Nutr.* **94:** 302–308.
10. Moore, T. 1976. Prolonged tests with retinoic acid as a source of vitamin A for rats. *J. Int. Vitaminol. Nutr.* **46:** 235–238.
11. Sani, B. P., and D. L. Hill. 1974. Retinoic acid: a binding protein in chick embryo metatarsal skin. *Biochem. Biophys. Res. Commun.* **51:** 1276–1282.
12. Ong, D. E., and F. Chytil. 1975. Retinoic acid-binding protein in rat tissue. Partial purification and comparison to rat tissue retinol-binding protein. *J. Biol. Chem.* **250:** 6113–6117.
13. Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature.* **330:** 444–450.
14. Giguere, V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid acid. *Nature.* **330:** 624–629.
15. Thompson, J. N., J. McC. Howall, and G. A. J. Pitt. 1964. Vitamin A and reproduction in rats. *Proc. R. Soc. London B.* **159:** 510–535.
16. De Ruyter, M. G., W. E. Lambert, and A. P. De Leenheer. 1979. Retinoic acid: an endogenous compound of human blood. Unequivocal demonstration of endogenous retinoic acid in normal physiological conditions. *Anal. Biochem.* **98:** 402–409.
17. De Leenheer, A. P., W. E. Lambert, and I. Claeys. 1982. All-*trans*-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J. Lipid Res.* **23:** 1362–1367.
18. Napoli, J. L., B. C. Pramanik, J. B. Williams, M. I. Dawson, and P. D. Hobbs. 1985. Quantification of retinoic acid by gas-liquid chromatography-mass spectrometry: total versus all-*trans*-retinoic acid in human plasma. *J. Lipid Res.* **26:** 387–392.
19. Barua, A. B., and J. A. Olson. 1986. Retinoyl β-glucuronide: an endogenous compound of human blood. *Am. J. Clin. Nutr.* **43:** 481–485.

20. Pearce, J. C., J. A. Jelly, K. A. Fernandes, W. J. Leavens, and R. D. McDowall. 1986. Analysis of 5-(4-acetamidophenyl)pyrazin-2(1H)-one (SK&F 94120) in plasma with an Analytichem automated sample processor liquid chromatography module. *J. Chromatogr.* **353:** 371–378.
21. Gelijkens, C. F., and A. P. De Leenheer. 1980. Reversed-phase liquid chromatography of 5-fluoro-uracil nucleotides in the presence of quaternary ammonium ions. *J. Chromatogr.* **194:** 305–314.
22. Eckhoff, Ch., B. Löfberg, I. Chahoud, G. Bochert, and H. Nau. 1989. Transplacental pharmacokinetics and teratogenicity of single dose of retinol (vitamin A) during organogenesis in the mouse. *Toxicol. Lett.* **48:** 171–184.
23. Frolik, C. A., P. P. Roller, A. B. Roberts, and M. B. Sporn. 1980. In vitro and in vivo metabolism of all-*trans*- and 13-*cis*-retinoic acid in hamsters. *J. Biol. Chem.* **255:** 8057–8062.
24. Brazzell, R. K., and W. A. Colburn. 1982. Pharmacokinetics of the retinoids isotretinoin and etretinate. *J. Am. Acad. Dermatol.* **6:** 643–651.
25. Wyss, R., and F. Bucheli. 1988. Quantitative analysis of retinoids in biological fluids by high-performance liquid chromatography using column switching. I. Determination of isotretinoin and tretinoin and their 4-oxo metabolites in plasma. *J. Chromatogr.* **424:** 303–314.
26. Curley, R. W., Jr., and J. W. Fowble. 1988. Photoisomerization of retinoic acid and its photoprotection in physiologic-like solutions. *Photochem. Photobiol.* **47:** 831–835.
27. Bhat, P. V., and P. R. Sundaresan. 1988. High-performance liquid chromatography of vitamin A compounds. *CRC Crit. Rev. Anal. Chem.* **20:** 197–219.